

IN THE SPECIFICATION:

Please replace the existing Title, with the following new title:
--METHOD OF FORMING VASCULARIZED KIDNEY TISSUE--.

Please replace the paragraph at page 1, beginning at line 11, with the following amended paragraph:

--Branching tubulogenesis is an essential mechanism by which epithelial tissues such as kidney, salivary gland and prostate develop (Proc. Natl. Acad. Sci. USA, 96, 7330-7335, 1999 incorporated herein by reference). Largely based on the classical studies of Brobstein and coworkers, direct interactions between mesenchymal and epithelial components of embryonic tissue have been thought to be crucial for branching morphogenesis in most epithelial tissues (1,2,3). During kidney development, for example, direct cell-cell interactions between the metanephric mesenchyme and the epithelial component, the ureteric bud (UB), are believed to be essential for branching morphogenesis of the latter (4,5,6). This view is based on the fact that it had not been possible, in many previous studies, to observe proliferation and branching of the UB in the absence of direct contact with the metanephric mesenchyme or another inducing tissue, suggesting that the developmental program necessary for branching depended upon direct contact between surface proteins of the UB with surface proteins of the metanephric mesenchyme. Further, no known soluble factor or set of factors had been able to induce UB branching morphogenesis *in vitro* (7).--

Please replace the paragraph beginning on page 2, line 3, with the following amended paragraph:

--This view has gained additional support from knockout experiments in which absent expression of a variety of individual soluble growth factors held to be important in kidney development, based upon previous organ culture experiments, fail to show defective branching morphogenesis of the UB (8,9). Nevertheless, recent studies have also shown that glial cell line derived neurotrophic factor (GDNF) is necessary for early UB outgrowth (10,11,12,13,14,15), but on its own, it fails to promote proliferation and branching morphogenesis of isolated UB in vitro. These results left open the possibility that some unknown soluble factor, or combination of factors, derived from the metanephric mesenchyme, might be sufficient to induce epithelial branching morphogenesis.--

Please replace the paragraph beginning on page 3, line 12, with the following amended paragraph:

--Recent advances in the molecular biology of kidney development demonstrate that specific molecular defects can explain a variety of clinical syndromes. VUR, UPJO, and various dysplastic, and hypoplastic kidney disorders have been known to co-exist and to be expressed in various human lineages with a variable penetrance, the so-called CAKUT syndrome. In addition, several targeted gene-deletion experiments have resulted in phenotypes that may be best characterized as resulting from defective UB morphogenesis (directly or indirectly). These range from the renal aplasia associated with complete UB failure associated with deletions of WT-1 and RTK c-ret molecules to more subtle effects resulting in hypoplasia or oligonephronia such as seen with certain integrin knockouts (34,46). Defective collecting system development may play a role in the most common congenital cystic disease, ADPKD. The inventors have shown that expression of PKD-1 correlates

spatiotemporally with branching morphogenesis of the UB (24). Findings such as this have led to the hypothesis that ADPKD and other cystic diseases of the kidney result from defects in the developmental program necessary for proper tubulogenesis.--

Please replace the paragraph beginning at page 11, line 17, with the following amended paragraph:

--Figure 2: A novel culture system for in vitro branching morphogenesis of the ureteric bud (UB) (Proc. Natl. Acad. Sci., 96, 7330-7335, 1999 incorporated herein by reference – please refer to this paper for color reproductions). UBs free from mesenchyme were micro-dissected from E-13 rat kidney rudiments and placed in an ECM gel suspension composed of type I collagen and growth factor-reduced Matrigel®, and cultured in BSN cell-conditioned medium (BSN-CM) supplemented with 10% FCS and growth factors. Details are given elsewhere in the text. The cultured UB was monitored daily by microscopy. Shown in the figure is transwell insert (1), ECM gel (2), isolated UB (3), polycarbonate filter (4), and BSN-CM plus growth factor(s) (5).--

Please replace the paragraph beginning at page 17, line 8, with the following amended paragraph:

--Hepatocyte growth factor (HGF), the receptor for which is *c-met*, a RTK, has been known for some time to be able to induce the formation of branching tubular structures when MDCK cells (a renal epithelial cell line) are seeded in Type I collagen matrix gels. Without HGF, these cells develop into cystic structures, but in the presence of HGF, the cells form cytoplasmic processes which eventually develop multicellular branching chords and then into tubular structures. The inventors have previously demonstrated that the HGF-induced structures have apical-basolateral

polarity, as determined by immunofluorescence with antisera against marker proteins for apical and basolateral surfaces of polarized tubular epithelial cells (Dev. Biol., 159, 535-548, 1993). Thus, HGF is sufficient, in the setting of the appropriate three dimensional extracellular matrix, to produce polarized tubular structures similar to those existing in the differentiated collecting ducts (Dev. Biol., 160, 293-302, 1993; Dev. Biol. 163, 525-529, 1993, Proc. Natl. Acad. Sci., 92, 4412-4416). The inventors have also developed novel cell culture models for branching tubulogenesis using both mature collecting duct cells (22) and embryonic ureteric bud cells (46). The morphogenesis of embryonic UB cells is largely dependent upon growth factors other than HGF (46).--

Please replace the paragraph beginning on page 19, line 1, with the following amended paragraph:

--**Cells and conditioned media:** The BSN cell line was derived from day 11.5 mouse embryonic kidney metanephric mesenchyme originally obtained from a mouse line transgenic for the early region of SV-40/large T antigen. As described elsewhere, the BSN cells express the mesenchymal protein marker vimentin, but not classic epithelial marker proteins such as cytokeratin, ZO-1 and E-cadherin (46). Differences in the expression patterns of 588 genes in BSN cells have been analyzed by the inventors on commercially available cDNA grids (Am. J. Physiol.-Renal Physiol., 277, F:650-F663, 1999), and confirmed the largely non-epithelial character of BSN-cells, though it remains to be determined whether they are mesenchymal or stromal, or have characteristics of both cell types. The SV-40/large T antigen transformed UB cell line and murine inner-medulla collecting duct (mIMCD) cells have been extensively characterized before (46, 47, 48, 49). To obtain conditioned media, a confluent cell monolayer was washed with serum-free medium, and then cultured in serum free medium for another 2-4 days. Various conditioned media were harvested after low speed centrifugation to remove cell debris and the concentrated 10-fold with a Centricon filter with 8 kDa nominal molecular weight cutoff (Millipore, Bedford, MA). In addition, BSN-CM was subfractionated on a heparin-sepharose affinity column (Hitrap Heparin; Pharmacia,

NJ). Concentrated BSN-CM (~10X) was applied to a heparin column. After washing the column with Hanks' balanced buffer solution, the heparin bound fraction was eluted with 2 M NaCl in Hanks' balanced buffer solution. After desalting with a PD-10 column (Pharmacia, NJ), the heparin bound fraction's final volume was adjusted to the starting volume. The heparin flow through fraction was collected and its volume was adjusted to the starting volume using a Centricon filter (8kDa cutoff). The partially purified fractions were assayed for their effect on UB morphogenesis in the presence of GDNF.--

Please replace the paragraph beginning at page 20, line 2, with the following amended paragraph:

--**The ECM gel mix:** The ECM gel mix was composed of 50% type I collagen (Collaborative Biomedical Product) and 50% growth factor-reduced Matrigel® (Collaborative Biomedical Product). The procedure for gelation has been previously described in detail (46) and is incorporated herein.--

Please replace the paragraph beginning on page 20, line 16, with the following amended paragraph:

--**Lectin staining:** 1) Dolichos Bioflorus (DB) lectin: Tissues were fixed with 2% paraformaldehyde for 30 minutes at 4 °C, permeabilized with 0.1% Saponin and then incubated with fluorescent conjugated DB (50 ug/ml, Vector) in a moisturized chamber for 60 minutes at 37 °C. After extensive washing, tissues were post-fixed in 2% paraformaldehyde again for 5 minutes and viewed using a laser scanning confocal microscope. The specificity of DB lectin binding has been demonstrated previously (20). 2) Peanut agglutinin (PNA) lectin: Tissues were fixed with 2% paraformaldehyde for 30 minutes at 4 °C; blocked with 50 mM NH₄Cl overnight at 4 °C, followed by an incubation with 1% gelatin in 0.075% Saponin for 30 minutes at 37 °C (24). After two washes with Neuraminidase buffer (150 mM NaCl, 50 mM Na-Acetate, pH 5.5), tissues were incubated with Neuraminidase (1 U/ml) for 4 hours at

37 °C and then with Rhodamine-conjugated PNA (50 ug/ml) for 60 minutes at 37 °C. Tissues were post-fixed with 2% paraformaldehyde and viewed with a laser scanning confocal microscope.--

Please replace the paragraph beginning at page 22, line 1, with the following amended paragraph:

--Immortalized UB cells have been shown by the inventors to undergo impressive morphogenesis in the presence of soluble factors (16) when seeded in extracellular matrix gels containing Type I collagen mixed with growth factor-depleted Matrigel®, a basement membrane extract derived from EHS sarcoma cells (Proc. Natl. Acad. Sci., 86, 7330-7335, 1999 incorporated herein by reference). A conditioned medium elaborated by BSN cells (BSN-CM), an immortalized line derived from early metanephric mesenchyme that has been developed by the inventors, has been shown to induce the formation of branching tubular structures, some of which have apparent lumens (16); the key activity in BSN-CM was shown to be distinct from a number of growth factors known to induce morphogenesis in mature kidney epithelial cell lines. The results from these cell culture studies suggest that the program for branching morphogenesis exists within UB cells and does not require direct contact with metanephric mesenchymal cells. Reasoning that the conditions for branching morphogenesis of isolated UB tissue might be similar to this in vitro cell culture system employing a UB cell line, the inventors separated embryonic rat kidney UB from the metanephric mesenchyme prior to induction and cultured the isolated UB (free from mesenchyme) in a mixture of collagen and growth factor deleted Matrigel® (Fig. 2). After trying many different conditions, dichotomous branching morphogenesis resembling the structures of the developing embryonic kidney was achieved when the isolated UB was cultured in the presence of a combination of BSN-CM and a mixture of growth factors (EGF, HGF, IGF, FGF-2 and GDNF) (Fig. 3). The growth factor mixture was chosen based upon the effects of individual factors on in vitro morphogenesis of cultured UB and mIMCD cells previously performed by the inventors (16, 22, 23, 24); HGF and EGF induce complex morphogenetic changes in UB and mIMCD cells, while IGF and FGF-2 induce some

morphogenetic changes in UB cells. Because of strong genetic and cell culture data supporting the role of GDNF/cRET in early UB morphogenesis and survival of UB-derived cells (~~11, 12, 25~~), GDNF was also added to the mixture.--

Please replace the paragraph beginning on page 23, line 4, with the following amended paragraph:

--At gestational day 13, rat UB is a "T" shaped epithelial tubule (Fig. 3a). *In vivo*, this single branched epithelial tubule undergoes repeated dichotomous branching and forms the "tree" shaped collecting system through interactions with metanephric mesenchyme (~~26~~). This epithelial-mesenchymal interaction is thought to be required for the tubular/ductal development of several organ systems, such as lung, pancreas and mammary gland (~~27, 28, 29~~). In the inventors' system, isolated UB (free from metanephric mesenchyme) can be cultured and induced to undergo branching morphogenesis *in vitro*. The cultured UB branched dichotomously with formation of structures that had apparent lumens. Each branch had both tubular and ampullary portions (Fig. 3b through d). Staining with lectins and antibodies indicated that the tubular structures remained UB-derived and epithelial in character. Both cell proliferation and branching morphogenesis appeared to occur simultaneously. In most cases, after 48 hours of culture, UB epithelial tissue started to increase in size and developed small protrusions from the "T" shaped ureteric bud. After 3-4 days of culture, those protrusions started to elongate, and the tips of the elongated structure started to branch dichotomously. The structures formed from the cultured UB revealed no staining with vimentin antibodies and peanut lectin (PNA), markers for mesenchymally derived elements, further supporting the notion that, in the appropriate milieu of soluble factors, complex branching of the UB can occur in the absence of direct contact with the metanephric mesenchyme. Moreover, growth of isolated UB was observed for up to 3-4 weeks, with many generations of branching.--

Please replace the paragraph beginning at page 24, line 14, with the following amended paragraph:

--Nevertheless, the factor (or a set of factors) in BSN-CM was not sufficient to induce UB branching morphogenesis. In the absence of the growth factor mixture, the UB underwent apoptosis as determined by the TUNEL assay (data not shown). To further define conditions for *in vitro* UB branching morphogenesis, the Inventors examined whether any single growth factor present in the growth factor mixture could, in combination with BSN-CM, induce UB branching morphogenesis. The combination of BSN-CM and GDNF, but no other combination, was found to be sufficient to induce the formation of three-dimensional branching structures comparable to those observed with BSN-CM and the growth factor mixture (Fig. 6A-D). Consistent with this observation, the combination of BSN-CM and GDNF prevented the UB from undergoing apoptosis and facilitated UB proliferation (data not shown). Since GDNF alone could not induce branching morphogenesis in the absence of BSN-CM, a factor or factors present in the BSN-CM must be required for the action of GDNF in the induction of UB branching morphogenesis. While studies from others have indicated that GDNF is involved in the initial formation of the UB (30,34), it has not been established whether GDNF is required for the further branching morphogenesis of the UB. Therefore, the UB was first cultured in the presence of BSN-CM and GDNF, and then in the absence of GDNF after repeatedly washing away GDNF from the culture and then adding antibodies known to neutralize GDNF in the system (Fig. 7A-C). Withdrawal of GDNF from the culture system blocked further UB branching morphogenesis, suggesting that GDNF is not only involved in early UB formation but also in further iterations of UB branching (Fig. 7D-F and compare Figs. 7E and 7F with Figs. 3b and 3c). In this regard, it is interesting to note that mesenchymal cell contact or some other soluble factor may be able to partially compensate for GDNF, at least under certain conditions in whole organ culture, since *c-RET* antisense oligonucleotides are not strongly inhibitory of continued branching of the UB when added after induction (32).--

Please replace the paragraph beginning at page 26, line 12, with the following amended paragraph:

--Thus, by utilizing the Inventors' novel model system, the Inventors have found that, in contrast to the widely held view that the complex arborization of the UB during kidney development is dependent upon direct contact between cells of the metanephric mesenchyme and cells of the UB, a substantial degree of branching morphogenesis can be mediated by soluble factors alone. Therefore, the branching program exists within the UB itself after it is formed from the Wolffian Duct, and soluble factors can trigger its initiation and continuation. No singular soluble factor, however, appears sufficient. A combination of GDNF and an activity, or set of activities, present in BSN-CM is necessary. Whether this latter activity is the same as that which induces the formation of branching tubules with lumens of UB cells in culture remains to be determined (16). It seems very likely that more direct mesenchymal interactions with the UB are important for establishing the direction of branching events since the cultured UB-derived structures lack directionality, and only when the culture UB was recombined with metanephric mesenchyme did directionality and elongation occur (Fig. 8H-I). Epithelial-mesenchymal cell-cell contact is probably essential for the later steps in the development of UB/collecting system. Additional mechanisms are likely to be involved in the formation of junctions between mesenchymally-derived nephronal segments and collecting tubules and the development of tertiary structures of the collecting tubule, such as the formation of arcades. Moreover, contact with the mesenchyme might provide a "stop" mechanism for kidney growth since Inventors found that the isolated UB continued to grow *in vitro* as long as soluble factors were provided.--